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MODELING AND STATISTICAL ANALYSIS OF BIOASSAY DATA: MEDAKA CELL PROLIFERATION UNDER DEN AND TCE

Donald P. Gaver
Patricia A. Jacobs

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NAVAL POSTGRADUATE SCHOOL
MONTEREY, CA 93943-5000

Rear Admiral T. A. Mercer
Superintendent

Harrison Shull
Provost

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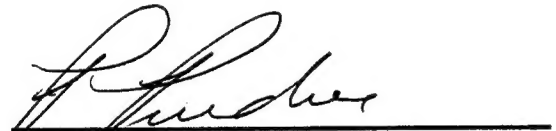
This report was prepared by:

DONALD P. GAVER, JR.
Professor of Operations Research

PATRICIA A. JACOBS
Professor of Operations Research

Reviewed by:



PETER PURDUE
Professor and Chairman
Department of Operations Research

Released by:

PAUL J. MARTO
Dean of Research

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The response of *medaka* liver to the chemicals DEN and TCE is analyzed statistically. The analysis illustrates the application of methods useful in *environmetrics*, i.e. environmental statistics. It suggests an overall dose-response *effect* but not an easily-interpreted dose-response functional *relationship*.

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Modeling and Statistical Analysis of Bioassay Data: Medaka Cell Proliferation under DEN and TCE

by

Donald P. Gaver

and

Patricia A. Jacobs

Department of Operations Research
Naval Postgraduate School
Monterey, CA 93943

1. INTRODUCTION

This report describes an analysis of cell proliferation data, by liver slice, from an experiment using Japanese Medaka. Previous work has used summary data from the same experiment (Gaver and Jacobs, 1994a,b). Another relevant reference is to Morris (1993). A brief description of the experiment follows.

The medaka are exposed to differing levels of DEN and TCE in tanks of water. The treatment groups are: control, 10 mg/ℓ DEN, 100 mg/ℓ DEN, 0.1 mg/ℓ TCE, (10 mg/ℓ DEN with 0.1 mg/ℓ TCE), 1 mg/ℓ TCE, and (10 mg/ℓ DEN with 1 mg/ℓ TCE). Each treatment group has two replicate tanks. Eight animals in each tank were sacrificed on 4 August, 1993; this is sacrifice B. Eight additional animals in each tank were sacrificed on 20 August 1993; this is sacrifice D.

Each sacrificed fish was exposed to BrdU for 72 hours prior to sacrifice; any cell that is in *S*-phase during this time has a BrdU marker. Each sacrificed fish is frozen and sliced longitudinally into 7-micron sections. A third of the slices are stained with another agent. This agent stains nuclei with the BrdU marker black; these nuclei are called *positive*. It is 5 of the latter stained slices that are analyzed subsequently.

Five slices containing a portion of the liver are considered for each fish. A region of interest (ROI) is marked on the slice; the ROI is chosen to attempt to maximize the number of hepatocytes and minimize the number of nonhepatocytes present. The area of all of the hepatocytes within the region of interest is measured, and the area of positive nuclei within the region of interest is measured. The number of hepatocytes in the ROI, and the number of positive hepatocytes in the ROI were also counted for half the fish in sacrifice B.

A count measure of cell proliferation, the *count index* (CI), for a slice is the number of positive hepatocytes in the ROI divided by the number of hepatocytes in the ROI, multiplied by 100. Evaluation of this measure is very labor intensive. As an alternative, the following *area index* (AI) is used

$$\text{AI} = \text{Area Index} = \frac{\text{Area of positive nuclei in the ROI}}{\text{Area of hepatocytes in the ROI}} \times 100.$$

The area index is easier to obtain; however, it does not quantify cells in *S*-phase exactly as does the CI since cells are of different size, as are the areas resulting from the slicing process, which are the result of a random intersection with the cell.

Figure 1 displays a plot of the count index (divided by 100) versus area index (divided by 100), computed by slice, for those fish of sacrifice B for which both measures are available, along with a simple unweighted least-squares-fitted

straight line; also displayed is the least-squares line equation with the standard errors of the coefficients displayed in parentheses below the coefficients. There appears to be a satisfactory linear relationship between the count index and area index, indicating that AI and CI are generally measuring the same response. However the variability of the area index increases as the count index increases; this increase is generally associated with high DEN and TCE dose levels; its biological interpretation is not yet available. It suggests that cell sizes become more variable under dosage.

Figure 2 is a display of the slice area indices (divided by 100), by fish, for the two control tanks. Note the variability between fish and the somewhat greater variability between fish in tank 2 as compared to those in tank 1.

Several slice area datum appear to be missing or are of doubtful validity. These have been deleted from analytical consideration. They are listed in Table 1. An alternative might have been to use robust statistical procedures throughout; such procedures automatically down-weight highly discrepant observations. Furthermore, examination of the weights indicates discrepancy so that explanations can be sought. It seems likely that robust methods should be more widely used in environmental toxicology. Robust statistical methods are discussed seriously in Cox and Hinkley (1974). A less advanced treatment appears in Koopmann (1987).

A summary of the findings of the data analysis is as follows.

a. Available data from sacrifice B suggests that there is a reasonably strong linear association between the count index and the area index. For ease of analysis the area index has been used throughout.

TABLE 1

Slices Not Considered in the Data Analysis

MISSING SLICES: SACRIFICE B

Missing Measurements are left blank.

ID	No. of Slices	Tank	Treatment	Reason
9273802	All	8	0.1 TCE	Blank field
9273811	All	9	10 DEN, 0.1 TCE	Blank field

MISSING SLICES: SACRIFICE D

Missing Values appear to be coded with 0.

ID	No. of Slices	Tank	Treatment	Reason
9273986	All	2	Control	Area ROI = Positive Area = 0
9285049	All	10	1 TCE	Area ROI = Positive Area = 0
9274001	All	4	10 DEN	All positive areas = 0 with 2 slices having 1 mit. hep.
9285079	All	14	10 DEN, 1 TCE	All positive areas = 0 with 3 slices having 1 mit. hep.
9285022	1	7	0.1 TCE	Area of ROI = 247.16, others of order 7500
9285043	1	9	10 DEN, 0.1 TCE	Positive area of slice = 0; 0 mit. cells
9285073	1	13	10 DEN, 1 TCE	Positive area of slice = 0; 0 mit. cells

b. There is evidence that the variances of the slice area indices over fish exposed to various treatments are approximately equal to the corresponding means. This relationship can lead to misleading conclusions if standard statistical procedures are used uncritically; furthermore, the results are less efficient than necessary. However, the variances of the *square roots* of the slice area

indices for fish subjected to the various treatment (DEN and TCE) levels appear approximately constant, i.e. far less dependent on the corresponding mean values. Consequently, the square roots of the area indices are used in subsequent data analyses. The underlying reason for the above data behavior is that positive counts are rare random events, hence tend to be approximately Poisson distributed. The square root transformation is known to stabilize the variance of such counts; see Miller (1986), p. 59. The same transformation should, and here does, stabilize the variance of count-associated areas.

c. The means of the fish mean square root of area indices are considered for both sacrifices. There is generally more treatment effect for sacrifice B than for sacrifice D: examine p-values in lines 3 of Tables 2 and 3 for the overall analysis of variance indications to see that sacrifice D p-values are always larger than those for sacrifice B. Four out of five of the treatment means are significantly larger (at the 95% level) than that for the control for sacrifice B. There is no significant difference (95% level) between the treatment means and the control mean for sacrifice D. For sacrifice B, the treatment means for two out of the three levels of TCE with 10 mg/ℓ DEN are significantly larger than those without DEN for sacrifice B; there is no significant difference for sacrifice D.

d. The control mean for sacrifice D is significantly larger than the control mean for sacrifice B. The other treatment means for sacrifice D are not significantly different than those for sacrifice B.

e. There is some suggestion that in the later sacrifice D, the presence of TCE *lowers* the mean of the area index. The biological mechanisms likely to explain this behavior are not yet available.

The above results are from one-way analysis of variance (ANOVA) of the square roots of the area indices, augmented by *multiple comparison* methods; the latter allow all possible pairwise comparisons to be made with a specified experiment-wise error rate, here 5%.

Brief Overall Summary of Findings to Date

The above can be briefly, and simplistically, summarized as follows. While it can be said that there is a statistically significant difference between mean responses ($\sqrt{\text{AI}}$) to the various treatments with DEN and TCE, no simple and interpretable dose-response patterns have been found. In particular, response does not appear to increase (or decrease) systematically with dose increase, where "dose" includes time of exposure as well as increases in chemical concentration dose levels. It remains to be seen whether the latter inconclusivity is lessened by the analysis of more data (later sacrifices), by finding that experimental problems or biases occurred, *or*, more exciting, that the dose-responses observed can be explained by biological mechanism, and that the findings essentially reappear when further experiments and data analyses are conducted.

Section 2 presents results of graphical displays of the data. Section 3 presents results of exploratory analyses of variance. Results of exploratory linear regression models are presented in Section 4. Multiple comparison results appear in Section 5.

2. GRAPHICAL SUMMARIES OF THE SLICE AREA INDICES

Figure 3a (respectively 3b) displays boxplots of the slice area indices by tank for sacrifice B, (respectively sacrifice D). The boxplots may be viewed as a graphical one-way analysis of variance. There is some tank effect within a treatment group. The greatest dose-response effect is clearly for the 100 DEN treatment.

Figures 4a and 4b display plots of the mean of the area indices for each tank versus the variance of the area indices for each tank. Also displayed is a 45° line. There appears to be a linear relationship between the mean and variance; in fact, the variances appear to be approximately equal to the means. This relationship between the means and variances may lead to misleading results if analysis of variance techniques are applied directly to the slice area indices; Miller (1986) and Box (1954) discuss the effects of inequality of variance on one-way analysis of variance. It is believed that such effects may well appear often in environmental toxicology, particularly where *counts*, or count-like phenomena, are found.

As noted earlier, one standard transformation that can be applied to data with variances approximately equal to means to attempt to make the variance of the transformed data more nearly constant is the *square root transformation*; see Miller (1986). Figures 5a and 5b display plots of the mean of the square root of the area indices for each tank versus the variance of the square root of the area indices for each tank. The variances now appear unrelated to the corresponding means. Figures 6a and 6b display boxplots of the square roots of the slice area indices by tank. Note that the lengths (heights between quartiles) of the boxes are less variable than are those for the boxplots of the raw area indices themselves. In the remainder of this paper the square root of area indices will be used.

3. EXPLORATORY ANALYSES OF VARIANCE

Results of exploratory analyses of variances appear in Tables 2 – 4. The basic data are summaries of area indices and the square roots of area indices for each fish. Since the boxplots indicate that the 100 DEN treatment is associated with much larger area indices, the analyses of variances were done with and without 100 DEN. Table 2 shows the results by tank. Recall that small *p*-values will indicate tank, hence treatment, effect. The first row of the table indicates that the tank means of the fish mean area index are significantly different. However the second row of the table indicates that the tank means of the logarithms of the variance (log variance) of area indices for each fish are also significantly different. The graphical analysis has already indicated this difference. Now recall that one of the assumptions of analysis of variance is that the data come from populations with equal variance, so apply the square-root transformation. Rows 3 and 4 report results using the square roots of the area indices. Note that the results of row 4 indicate that there is no significant difference between the tank means of the log variance of the slice square root of area index for each fish. However, the results of row 3 indicate that there *is* significant difference between the tank *means* of the mean of the slice square root area indices for each fish; note that the *p*-values for the tank means are smaller using the fish mean square root of area indices rather than the raw (untransformed) area indices for the analysis of variance (without the 100 DEN treatment). Thus, the difference in variances of the raw (untransformed) area indices appears to have masked some of the difference in means, presumably resulting from treatment effects.

Table 3 displays results for analyses of variance, with the two tanks in each treatment combined. Once again, the analysis of variance using the mean slice

square root of the area indices for each fish indicates that there is a significant difference between treatment means of the means, even without 100 DEN.

TABLE 2

***p*-Values for Exploratory ANOVA of Area Indices (AI) by Tank**
(AI = [(Positive Area)/ROI Area] × 100)
(Small *p*-Values Indicate Chemical-Tank Effect)

	Sacrifice B		Sacrifice D	
Data	with 100 DEN	without 100 DEN	with 100 DEN	without 100 DEN
Mean slice AI for each fish in a tank	$< 10^{-16}$	3×10^{-5}	6×10^{-16}	2.9×10^{-2}
Log variance of slice AI for each fish in a tank	4×10^{-11}	2×10^{-5}	3×10^{-2}	0.54
Mean slice $\sqrt{AI}$ for each fish in a tank	4×10^{-16}	8×10^{-7}	1×10^{-14}	1.6×10^{-2}
Log variance of slice $\sqrt{AI}$ for each fish in a tank	0.16	0.25	0.89	0.97

TABLE 3

***p*-Values for Exploratory ANOVA of Area Indices (AI) by Treatment**
(AI = [(Positive Area)/ROI Area] × 100)
Treatment: 2 Tanks Combined
(Small *p*-Values Indicate Treatment Effect)

	Sacrifice B		Sacrifice D	
Data	with 100 DEN	without 100 DEN	with 100 DEN	without 100 DEN
Mean slice AI for each fish in a treatment	1×10^{-16}	1×10^{-5}	1×10^{-16}	2×10^{-2}
Log variance of slice AI for each fish in a treatment	4×10^{-13}	3×10^{-6}	1×10^{-4}	0.33
Mean slice $\sqrt{AI}$ for each fish in a treatment	2×10^{-16}	9×10^{-7}	4×10^{-16}	9×10^{-3}
Log variance of slice $\sqrt{AI}$ for each fish in a treatment	0.19	0.46	0.56	0.89

Table 4 displays results of an analysis of variance of the mean square root of area indices for each fish in a treatment but without the control. Once again there is evidence of significant differences between the treatment means.

We conclude that there is a definite treatment effect, i.e. response to different treatment levels, even if no treatment = control and the "strong" 100 DEN treatment responses are removed.

TABLE 4

***p*-Values for Exploratory ANOVA of Area Indices (AI) by Treatment**
Without Control
(AI = [(Positive Area)/ROI Area] × 100)
Treatment: 2 Tanks Combined
(Small *p*-Values Indicate Treatment Effect)

	Sacrifice B		Sacrifice D	
Data	with 100 DEN	without 100 DEN	with 100 DEN	without 100 DEN
Mean $\sqrt{AI}$ for fish	0	1.9×10^{-3}	5.9×10^{-15}	7.4×10^{-3}

4. EXPLORATORY LINEAR REGRESSION

Tables 5 – 8 report results of fitting exploratory linear regression models to the square root of the slice area indices for each fish. For Tables 5 and 6, the covariates are the level of DEN minus its mean; the level of TCE minus its mean; and an interaction term: {(level of DEN minus its mean) times (level of TCE minus its mean)}. The means were subtracted to give the interaction term a value other than 0 if the level of DEN or the level of TCE is 0. The linear regressions were fit with and without the 100 DEN treatment. The results of Table 5 are for sacrifice B and those of Table 6 are for sacrifice D.

TABLE 5

Sacrifice B: $\sqrt{AI}$
Linear Regression Coefficient Estimates with Standard Error and 95% Normal Confidence Intervals

CONSTANT (SE) [CI]	DEN-$\overline{DEN}$ (SE) [CI]	TCE-$\overline{TCE}$ (SE) [CI]	(DEN-$\overline{DEN}$)×(TCE-$\overline{TCE}$) (SE) [CI]	R^2 — —	s.e. — —
WITHOUT 100 DEN					
1.88	0.037	0.526	0.025	0.15	0.50
(0.067)	(0.005)	(0.148)	(0.010)		
[1.75,2.01]	[0.028,0.046]	[0.237,0.816]	[0.005,0.045]		
$\overline{DEN}$ = 5.0 $\overline{TCE}$ = 0.372					
WITH 100 DEN					
1.77	0.029	0.710	0.039	0.56	0.50
(0.040)	(0.003)	(0.117)	(0.008)		
[1.69,1.85]	[0.024,0.033]	[0.48,0.94]	[0.02,0.05]		
$\overline{DEN}$ = 18.81 $\overline{TCE}$ = 0.318					

TABLE 6

Sacrifice D: $\sqrt{AI}$
Linear Regression Coefficient Estimates with Standard Error and 95% Normal Confidence Intervals

CONSTANT (SE) [CI]	DEN-$\overline{DEN}$ (SE) [CI]	TCE-$\overline{TCE}$ (SE) [CI]	(DEN-$\overline{DEN}$)×(TCE-$\overline{TCE}$) (SE) [CI]	R^2 — —	s.e. — —
WITHOUT 100 DEN					
1.89	0.028	-0.402	-0.025	0.08	0.50
(0.07)	(0.005)	(0.151)	(0.01)		
[1.76,2.03]	[0.02,0.04]	[-0.70,-0.11]	[-0.05,-0.005]		
$\overline{DEN}$ = 4.88 $\overline{TCE}$ = 0.37					
WITH 100 DEN					
1.68	0.013	-0.050	0.0005	0.45	0.50
(0.04)	(0.003)	(0.12)	(0.008)		
[1.60,1.76]	[0.008,0.018]	[-0.29,0.19]	[-0.02,0.02]		
$\overline{DEN}$ = 19.05 $\overline{TCE}$ = 0.315					

In both tables the values of R^2 are small when the 100 DEN data are excluded. This implies that a linear function of the above explanatory variables does not explain the data well. However, the standard errors of the estimates are also small. This behavior suggests that, although there may be an association between levels of DEN and TCE and the square root of the area index, that association is not linear. Note that for sacrifice B all of the estimates of the coefficients are significantly positive, suggesting that increasing levels of DEN and TCE are associated with higher area indices. However, for sacrifice D, the estimate of the coefficient of the level of TCE and the estimate of the coefficient of the interaction term coefficient are significantly negative for the regression, even without the 100 DEN treatment. This suggests that for the later sacrifice, exposure to TCE may have an inhibitory effect. The only estimate of covariate that is significantly different from 0 for sacrifice D when 100 DEN is included is exposure to DEN.

Tables 7 and 8 report results of fitting linear regressions with the covariate being the level of TCE exposure to data from fish not exposed to DEN and to data from fish exposed to 10 mg/ℓ DEN; the dependent variable is the square root of the slice area index. The R^2 values are very small, indicating a lack of linear fit, but the estimate coefficients for TCE in the regressions using data from fish exposed to 10 mg/ℓ DEN are formally significant. Once again, this behavior suggests that there may be an association but the association is not linear.

For sacrifice B, there was no significant effect for the level of TCE for the fish not exposed to DEN; for those fish exposed to 10 mg/ℓ DEN the coefficient for level of TCE is significantly positive indicating that increasing levels of TCE are associated with increasing (square roots of) area indices.

TABLE 7

Sacrifice B: $\sqrt{AI}$
Linear Regression Coefficient Estimates with Standard Error and 95% Normal Confidence Intervals
(Replicate Tanks Pooled)

CONSTANT (SE) [CI]	TCE (SE) [CI]	DEN (SE) [CI]	R^2 — —	s.e. — —
NO DEN				
1.18	0.056	–	0.002	0.55
(0.047)	(0.080)	–		
[1.09, 1.27]	[-0.10, 0.21]	–		
10 DEN				
1.47	0.31	–	0.09	0.44
(0.037)	(0.06)	–		
[1.40, 1.54]	[0.18, 0.43]	–		
0 DEN and 10 DEN				
1.14	0.18	0.038	0.14	0.50
(0.038)	(0.051)	(0.005)		
[1.06, 1.21]	[0.08, 0.28]	[0.29, 0.047]		

In sacrifice D, for those fish not exposed to DEN, the estimate of the coefficient of TCE is not significantly different than 0. However, for those fish exposed to 10 mg/ℓ DEN the estimate of the coefficient of TCE is significantly *negative*, suggesting that for the fish of the later sacrifice that were exposed to DEN, the *greater* the level of TCE exposure, the *smaller* the (square root of) the area index. This effect calls for biological explanation.

TABLE 8

Sacrifice D: $\sqrt{AI}$
Linear Regression Coefficient Estimates with Standard Error and 95% Normal Confidence Intervals
(Replicate Tanks Pooled)

CONSTANT (SE) [CI]	TCE (SE) [CI]	DEN (SE) [CI]	R^2 — —	s.e. — —
NO DEN				
1.35	0.059	–	0.004	0.43
(0.037)	(0.062)	–		
[1.27, 1.42]	[-0.063, 0.181]	–		
10 DEN				
1.71	-0.189	–	0.02	0.56
(0.048)	(0.083)	–		
[1.61, 1.80]	[-0.352, -0.026]	–		
0 DEN and 10 DEN				
1.39	-0.061	0.027	0.07	0.50
(0.038)	(0.052)	(0.005)		
[1.32, 1.47]	[-0.16, 0.04]	[0.017, 0.036]		

5. MULTIPLE COMPARISONS

The exploratory analyses of variances strongly rejected the null hypothesis that all the treatment means (even without the 100 DEN treatment) of the fish mean square root of the area indices are equal. Rejection of the null hypothesis does not indicate specifically which means are not equal. A method for discovering which means differ is called a multiple comparisons procedure. There are a number of different *multiple comparisons* procedures in the literature; see Miller (1981). We will use two of them.

5.1 Simultaneous Confidence Intervals using Studentized Range Distribution

The first procedure uses the studentized range distribution to construct simultaneous confidence statements about the true values of all differences of the treatment means. Table 9 describes the procedure to obtain simultaneous 95% confidence intervals for all differences of treatment means for one sacrifice without the 100 DEN treatment. The original procedure requires that there be an equal number of fish in each treatment. However, Ott *et al.* suggest step 4 in Table 9 if the number of fish in each treatment do not differ by much.

5.1a Treatment Means Minus Control Mean

Figure 7 presents some of the 95% simultaneous confidence intervals of the differences of treatment means for sacrifice B. It shows the confidence intervals for the treatment means minus the control mean for the fish mean square root of the area indices. Note that 4 out of the 5 intervals are significantly above 0 indicating that the treatments are associated with a larger mean square root area indices than those for the control. The greatest difference is that for the treatment of 10 mg/ℓ DEN with 1 mg/ℓ TCE.

Figure 8 presents some of the 95% simultaneous confidence intervals for the later sacrifice D. It shows the confidence intervals for the treatment mean minus the control mean. None of the treatment means is significantly different from the control mean.

5.1b Treatment Means for Treatments with Exposure to 10 mg/ℓ DEN Minus Those without Exposure to 10 mg/ℓ DEN

Figure 9 displays the sacrifice B 95% simultaneous confidence intervals for the difference between the treatment means for mean fish square root of the area indices for those treatments *with* 10 mg/ℓ DEN minus the treatment means for those treatments *without* 10 mg/ℓ DEN, by level of TCE exposure. Note that the

treatment means with 10 mg/ℓ DEN is significantly larger than that without for 0 mg/ℓ TCE and 1 mg/ℓ TCE. There is no significant difference for 0.1 mg/ℓ TCE.

TABLE 9

To Obtain Tukey (Studentized Range Distribution) Simultaneous 95% Confidence Intervals for Treatment Mean Differences, One Sacrifice

1.	There are 6 treatments: Control; 10 DEN; 0.1 TCE; 10 DEN with 0.1 TCE; 1 TCE; and 10 DEN with 1 TCE.
2.	There are ~88 within degrees of freedom $\left(\sum_{i=1}^{6}(n_i-1)\right.$ where n_i is the number of fish in treatment $i\left.\right)$.
3.	The 0.05 percentage point for the studentized range for 60 within-degrees-of-freedom and 6 treatment means is 4.16, from published tables, BIOMETRIKA *Tables for Statisticians*, Vol. 1. This is larger than the percentage point for 88 within-degrees-of-freedom. Thus, the constructed confidence intervals will be conservative: one can truly say that ***all*** pairwise difference comparisons are made with (95%) confidence.
4.	Since the number of fish per treatment differs somewhat (due to mssing fish) the harmonic mean of the number of fish per treatment is used $$n=\frac{6}{\frac{1}{n_1}+\ldots+\frac{1}{n_6}}$$ where n_i is the number of fish in treatment i.
5.	The mean square within is $$\frac{\sum_{i=1}^{6}\sum_{j}\left(y_{ij}-\bar{y}_{i\cdot}\right)^2}{\sum_{i=1}^{6}(n_i-1)}=\text{MS(within)}$$ where y_{ij} is the mean$\sqrt{\text{AI}}$ for fish j in treatment i and $\bar{y}_{i\cdot}$ is the mean of the mean $\sqrt{\text{AI}}$ for the fish in treatment i.
6.	95% confidence intervals for all pairs of means μ_i and μ_i' $$(\bar{y}_{i\cdot}-\bar{y}_{i'\cdot})\pm(4.16)\sqrt{\text{MS(within)}/n}$$

Figure 10 displays a similar plot for the later sacrifice D. There is no significant difference between the treatment means with 10 mg/ℓ DEN and those without.

5.1c Treatment Means for Sacrifice D Minus Treatment Means for Sacrifice B

Simultaneous confidence intervals are computed for all differences of the treatment means of the fish mean square root of the area indices for sacrifices B and D combined. Figure 11 displays six of the 95% simultaneous confidence intervals. It displays the 95% confidence intervals for the difference in treatment means between sacrifice D and sacrifice B. The only significant difference is for the control where the mean of the fish mean square root of area indices for sacrifice D is significantly larger than that for sacrifice B.

5.2 Studentized Maximum Modulus Confidence Intervals

Simultaneous confidence intervals for the treatment means themselves can be constructed using the studentized maximum modulus procedure; cf. Miller (1981). The procedure is as follows for the mean fish square root area index for the three treatment groups (10 mg/ℓ DEN, 0 mg/ℓ TCE), (10 mg/ℓ DEN, 0.1 mg/ℓ TCE), and (10 mg/ℓ DEN, 1 mg/ℓ TCE) for 1 sacrifice.

To Obtain Simultaneous Confidence Intervals for 3 Treatment Means using the Studentized Maximum Modulus Distribution

1. Compute the within degrees of freedom for the three treatments.

$$d = \sum_{i=1}^{3} (n_i - 1)$$

where n_i is the number of fish in treatment i.

2. Compute the mean square within

$$\text{MS(within)} = \frac{\sum_{i=1}^{3}\sum_{j}\left(y_{ij} - \bar{y}_{i\cdot}\right)^2}{\sum_{i=1}^{3}(n_i - 1)}$$

where y_{ij} is the mean square root of the area indices for fish j in treatment i and $\bar{y}_{i\cdot}$ is the mean of the fish means in treatment i.

3. Find the upper 0.05 point of the studentized maximum modulus distribution with parameters 3 (treatments) and d degrees of freedom, $m(3, d)$. Tables can be found in Miller (1981).

4. The three simultaneous 95% confidence intervals are

$$\bar{y}_{i\cdot} \pm m(3,d)\sqrt{\text{MS(within)}/n_i}\,.$$

Figure 12 displays the 95% simultaneous confidence intervals for sacrifice B for the means of the fish mean of the square root of the slice area indices for those treatments having fish exposed to 10 mg/ℓ DEN. The means appear about the same for 0 mg/ℓ TCE and 0.1 mg/ℓ TCE. The mean for 1 mg/ℓ TCE appears to be somewhat larger.

Figure 13 displays the 95% simultaneous confidence intervals for sacrifice D for the means of those treatments with 10 mg/ℓ DEN by level of TCE. There is some suggestion that the presence of TCE is associated with a *lower* mean of the fish mean square root of the slice area indices.

CONCLUSION

The above analyses illustrate the use of statistical methods appropriate for the kinds of data obtained by the medaka experiments. The methods of

transformation, analysis of variance, and multiple comparisons are useful and powerful for the initial data analyses, suggesting some surprising dose-response relations that are worthy of careful further biological investigation and explanation. Alternative methods can also be applied, and should yield the same general insights.

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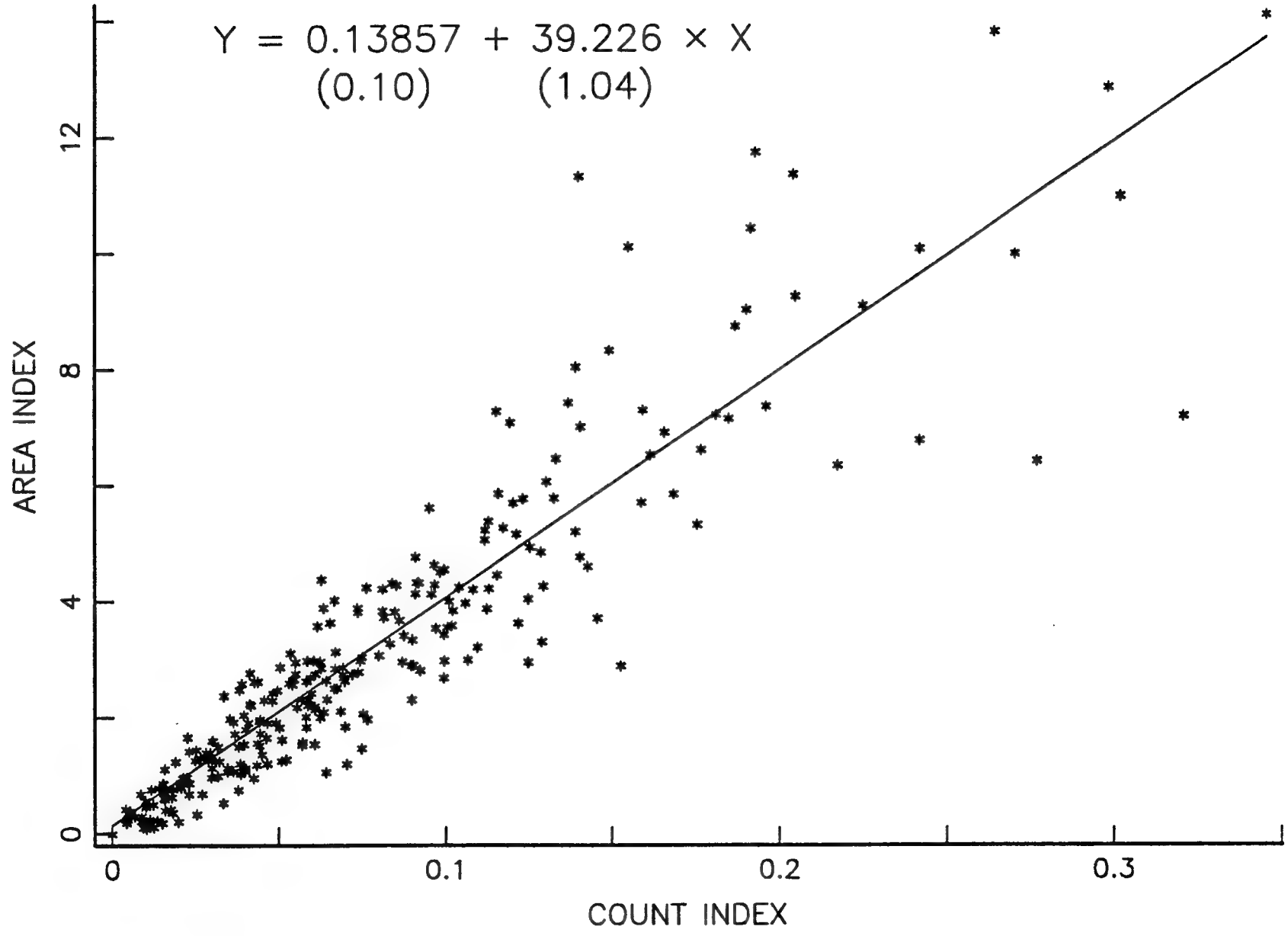
AREA INDEX VERSUS COUNT INDEX BY SLICE
SCATTER PLOT, SSZ=275
Y = 0.13857 + 39.226 × X
(0.10) (1.04)
AREA INDEX
0
4
8
12
0
0.1
0.2
0.3
COUNT INDEX

Figure 1

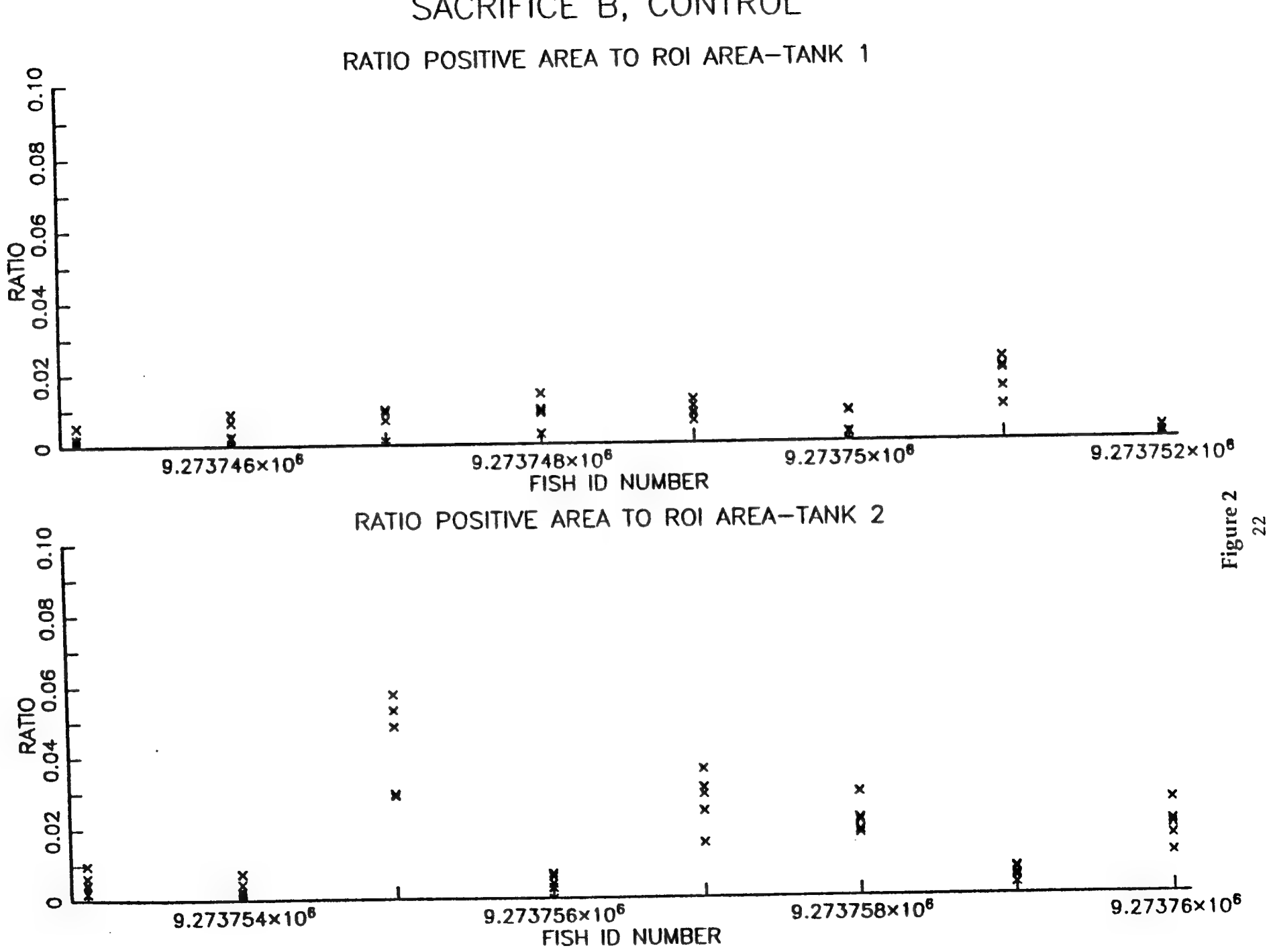
SACRIFICE B, CONTROL
RATIO POSITIVE AREA TO ROI AREA–TANK 1
RATIO
0
0.02
0.04
0.06
0.08
0.10
9.273746×10⁶
9.273748×10⁶
9.27375×10⁶
9.273752×10⁶
FISH ID NUMBER
RATIO POSITIVE AREA TO ROI AREA–TANK 2
RATIO
0
0.02
0.04
0.06
0.08
0.10
9.273754×10⁶
9.273756×10⁶
9.273758×10⁶
9.27376×10⁶
FISH ID NUMBER

Figure 2

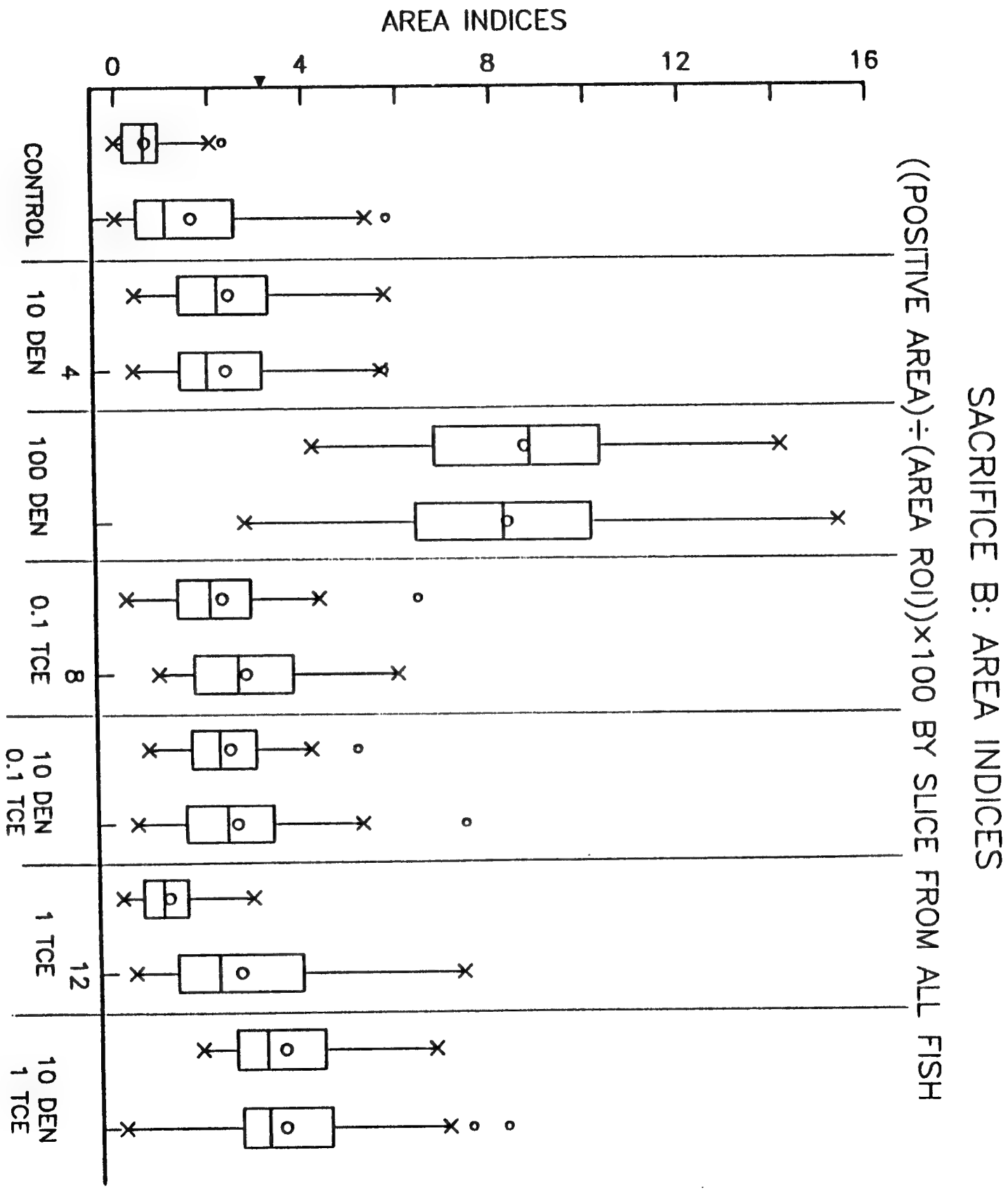
AREA INDICES
0
4
8
12
16
CONTROL
10 DEN
4
100 DEN
0.1 TCE
8
10 DEN
0.1 TCE
1 TCE
12
10 DEN
1 TCE
((POSITIVE AREA)÷(AREA ROI))×100 BY SLICE FROM ALL FISH
SACRIFICE B: AREA INDICES

Figure 3a

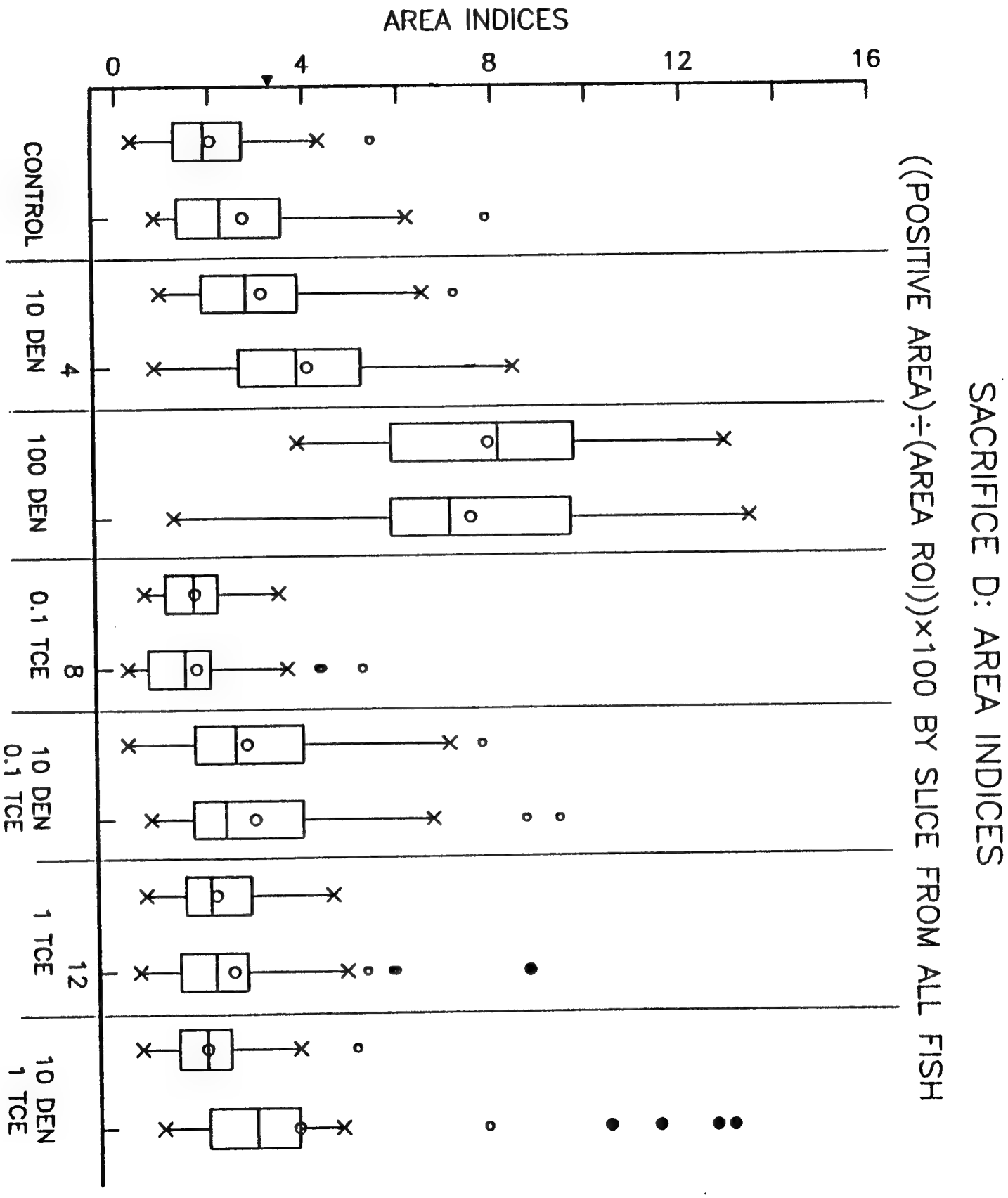
AREA INDICES
0
4
8
12
16
CONTROL
10 DEN
4
100 DEN
0.1 TCE
8
10 DEN
0.1 TCE
1 TCE
12
10 DEN
1 TCE
((POSITIVE AREA)÷(AREA ROI))×100 BY SLICE FROM ALL FISH
SACRIFICE D: AREA INDICES

Figure 3b

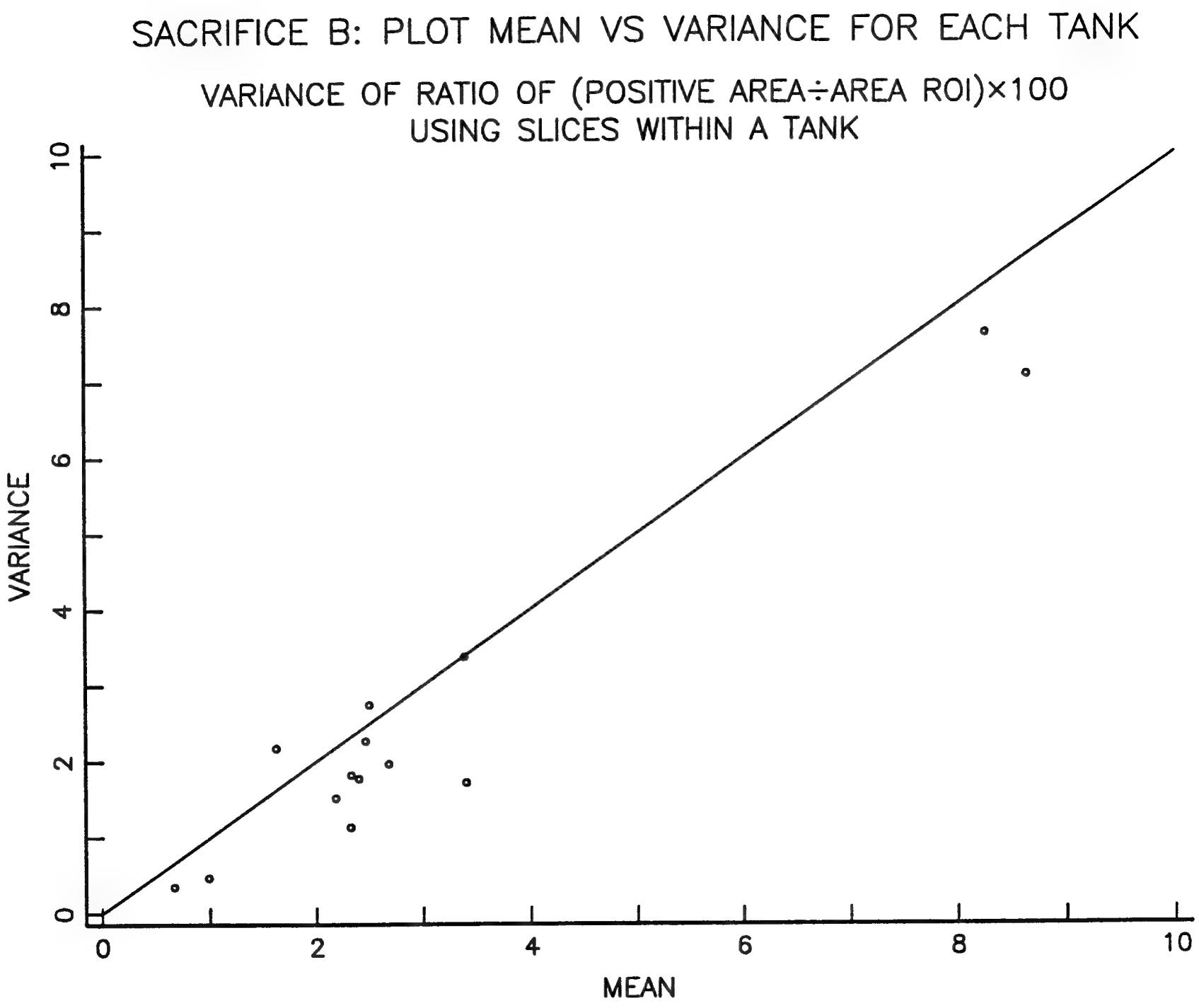
SACRIFICE B: PLOT MEAN VS VARIANCE FOR EACH TANK
VARIANCE OF RATIO OF (POSITIVE AREA÷AREA ROI)×100
USING SLICES WITHIN A TANK
VARIANCE
0
2
4
6
8
10
MEAN
0
2
4
6
8
10

Figure 4a

SACRIFICE D: PLOT MEAN VS VARIANCE FOR EACH TANK
VARIANCE OF RATIO OF (POSITIVE AREA÷AREA ROI)×100
USING SLICES WITHIN A TANK
VARIANCE
MEAN
0
2
4
6
8
10

Figure 4b

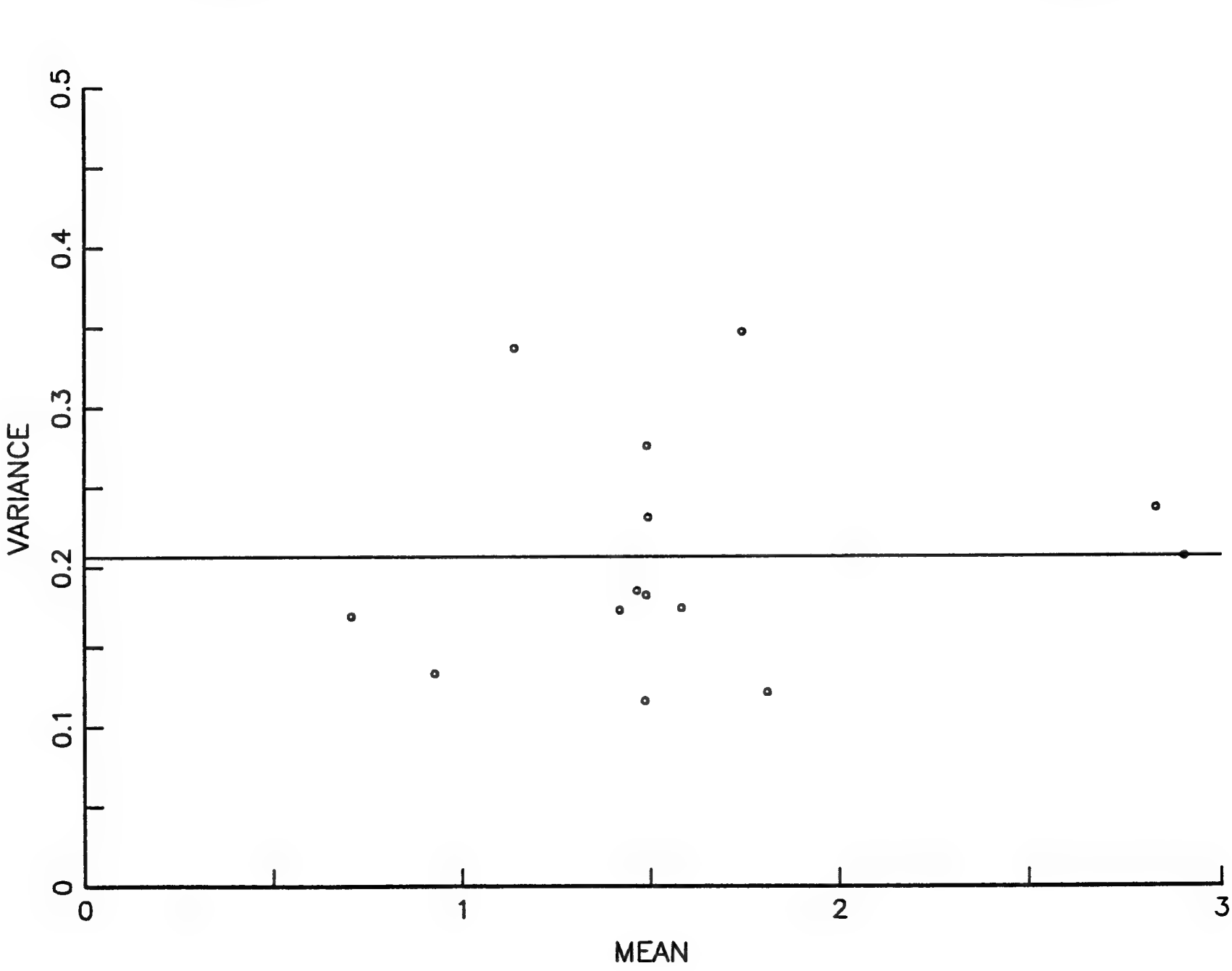
SACRIFICE B: SQUARE ROOT AREA INDEX BY TANK
VARIANCE
0
0.1
0.2
0.3
0.4
0.5
0
1
2
3
MEAN

Figure 5a

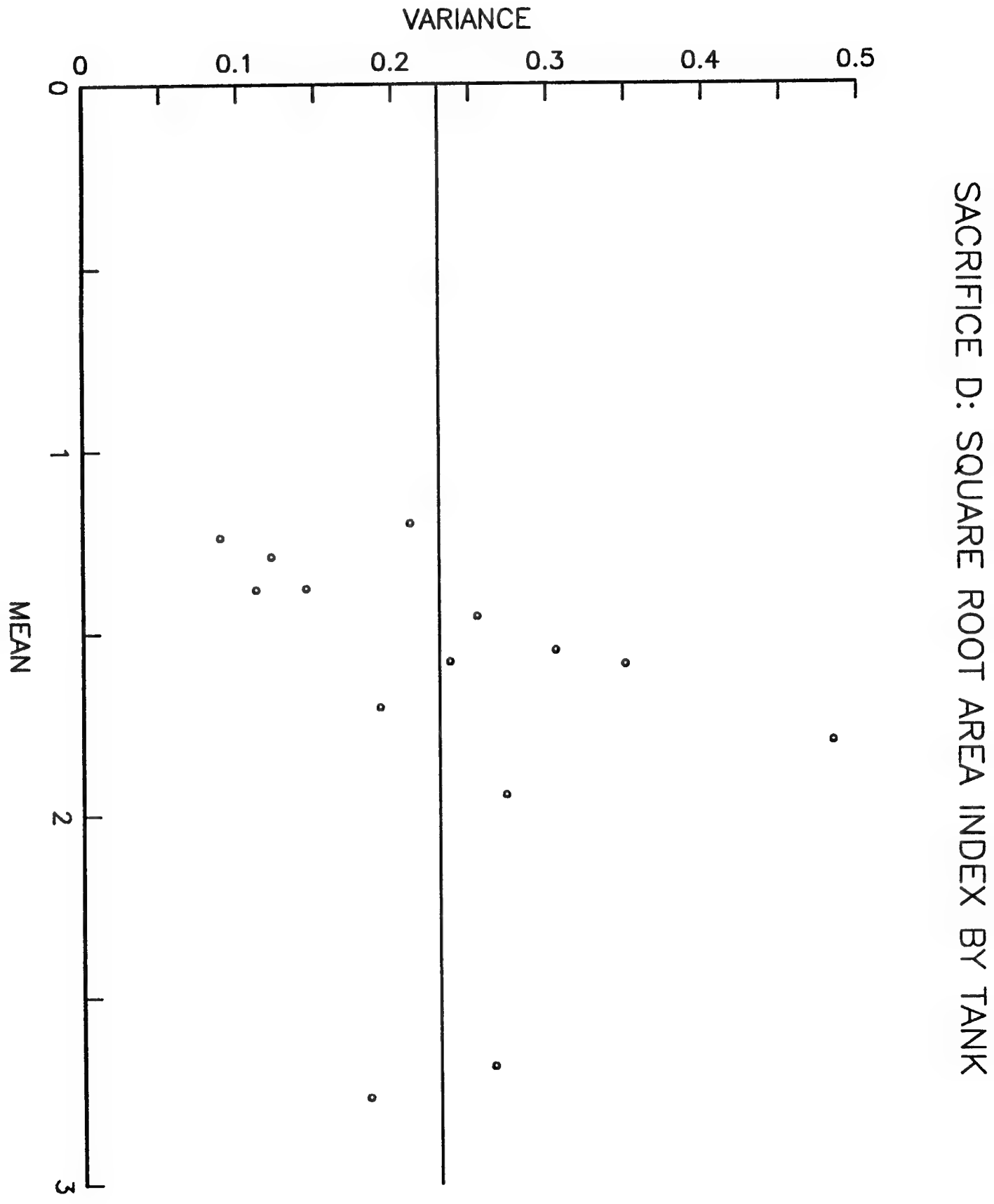
SACRIFICE D: SQUARE ROOT AREA INDEX BY TANK
VARIANCE
0
0.1
0.2
0.3
0.4
0.5
MEAN
0
1
2
3

Figure 5b

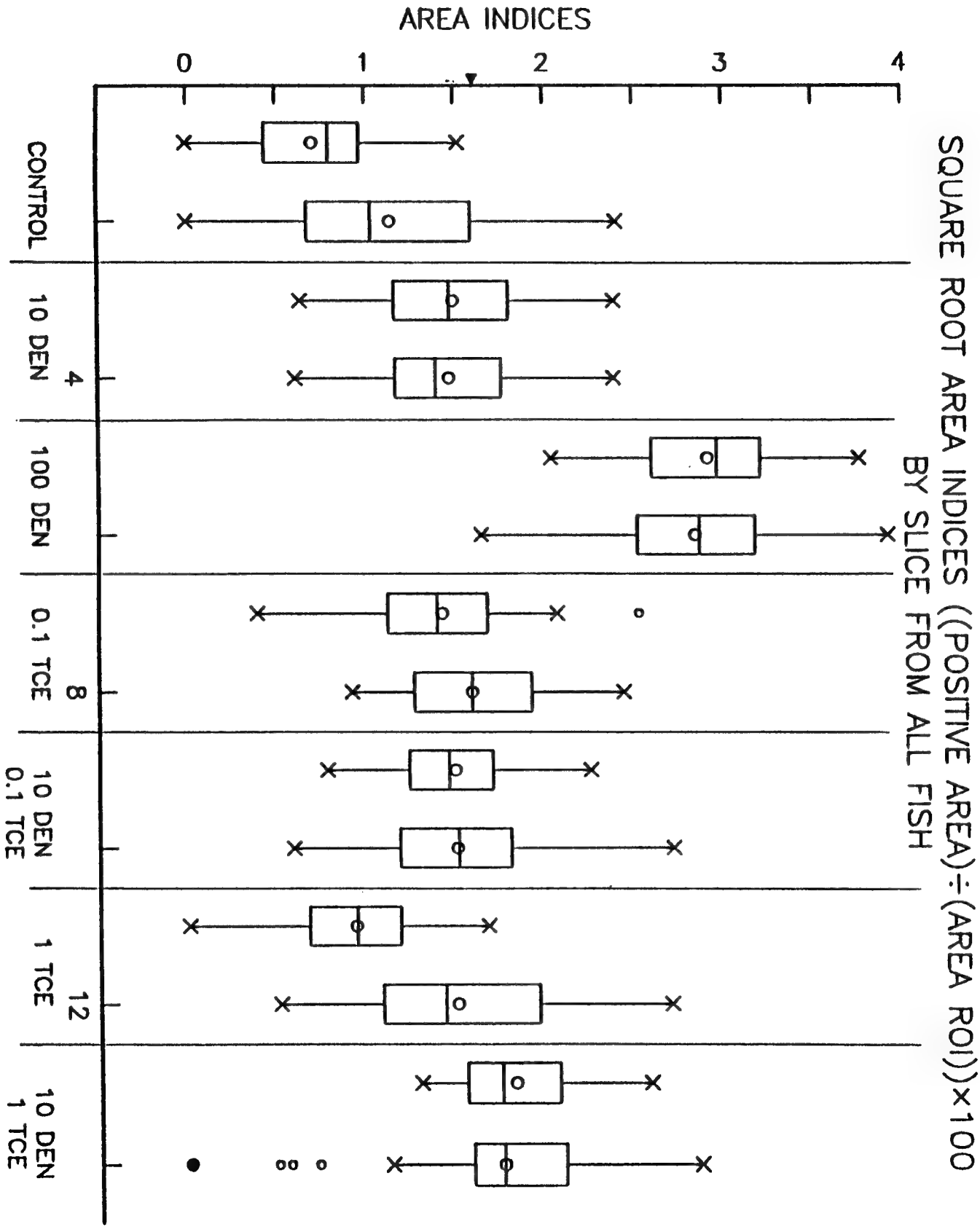
AREA INDICES
0
1
2
3
4
CONTROL
10 DEN
4
100 DEN
0.1 TCE
8
10 DEN
0.1 TCE
1 TCE
12
10 DEN
1 TCE
SQUARE ROOT AREA INDICES ((POSITIVE AREA)÷(AREA ROI))×100
BY SLICE FROM ALL FISH
SACRIFICE B

Figure 6a

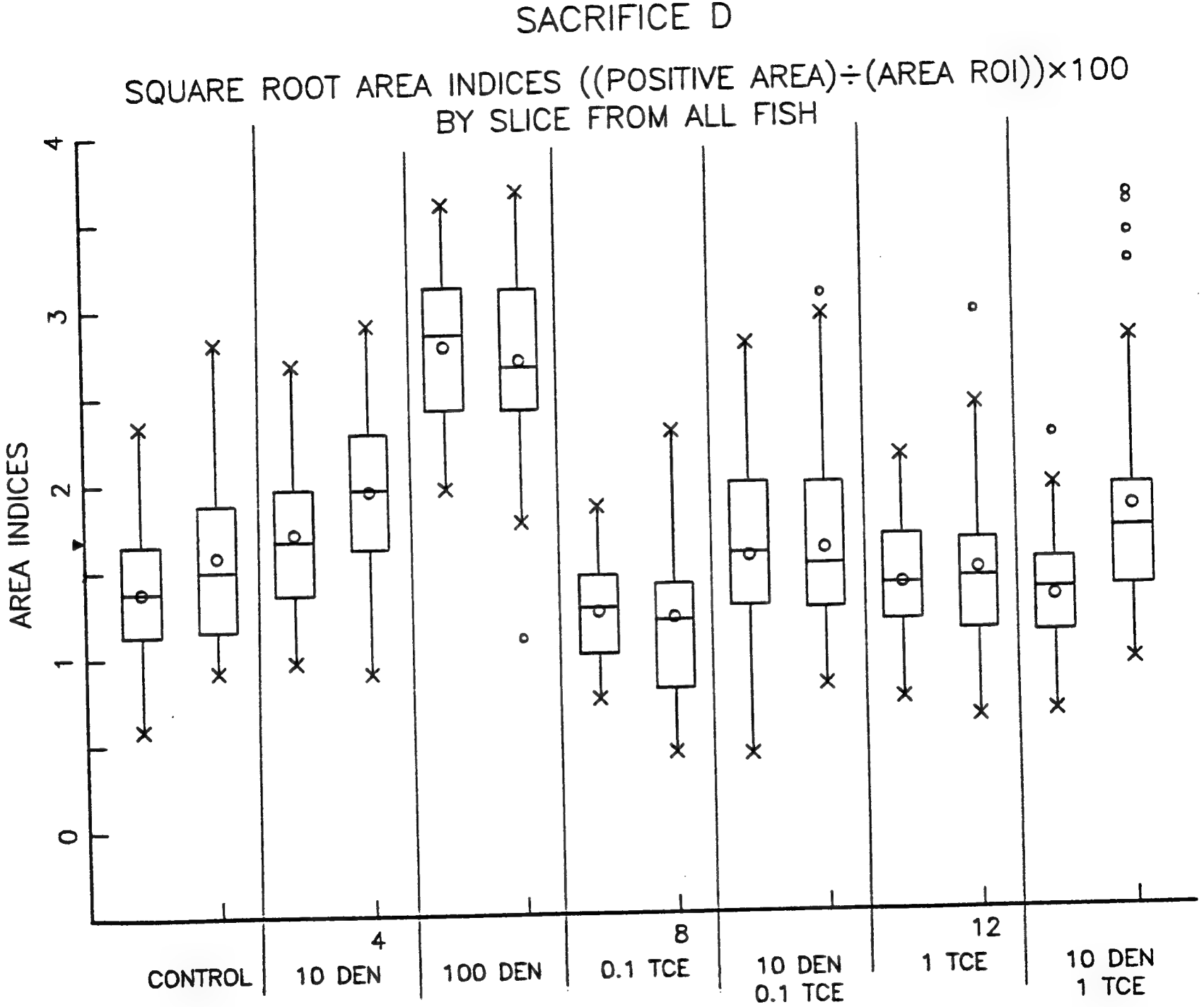
SACRIFICE D
SQUARE ROOT AREA INDICES ((POSITIVE AREA)÷(AREA ROI))×100
BY SLICE FROM ALL FISH
AREA INDICES
0
1
2
3
4
8
CONTROL
4
10 DEN
100 DEN
8
0.1 TCE
10 DEN
0.1 TCE
12
1 TCE
10 DEN
1 TCE

Figure 6b

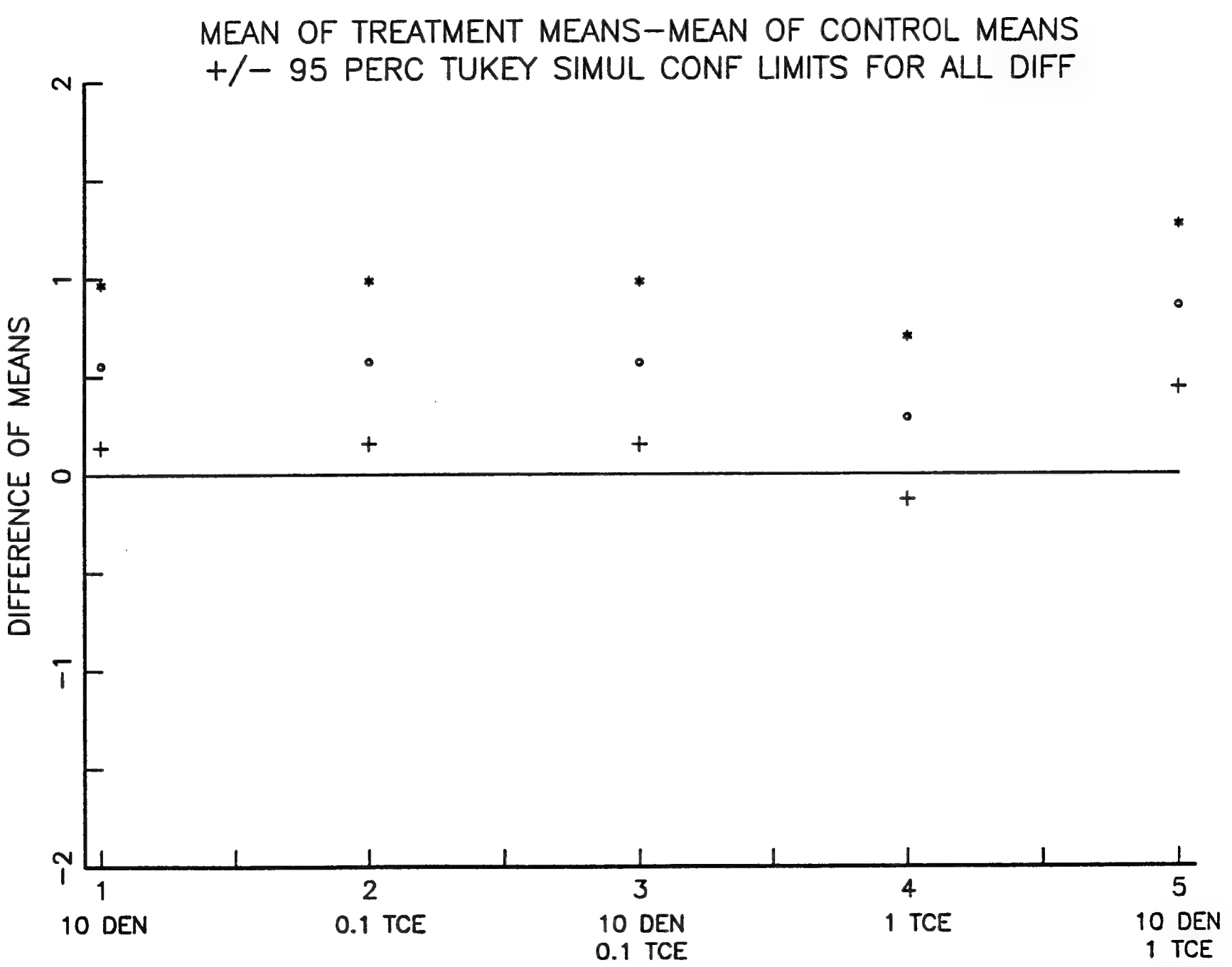
SACRIFICE B: SQUARE ROOT OF AREA INDICES
MEAN OF TREATMENT MEANS–MEAN OF CONTROL MEANS
+/– 95 PERC TUKEY SIMUL CONF LIMITS FOR ALL DIFF
DIFFERENCE OF MEANS
2
1
0
-1
-2
1
10 DEN
2
0.1 TCE
3
10 DEN
0.1 TCE
4
1 TCE
5
10 DEN
1 TCE

Figure 7

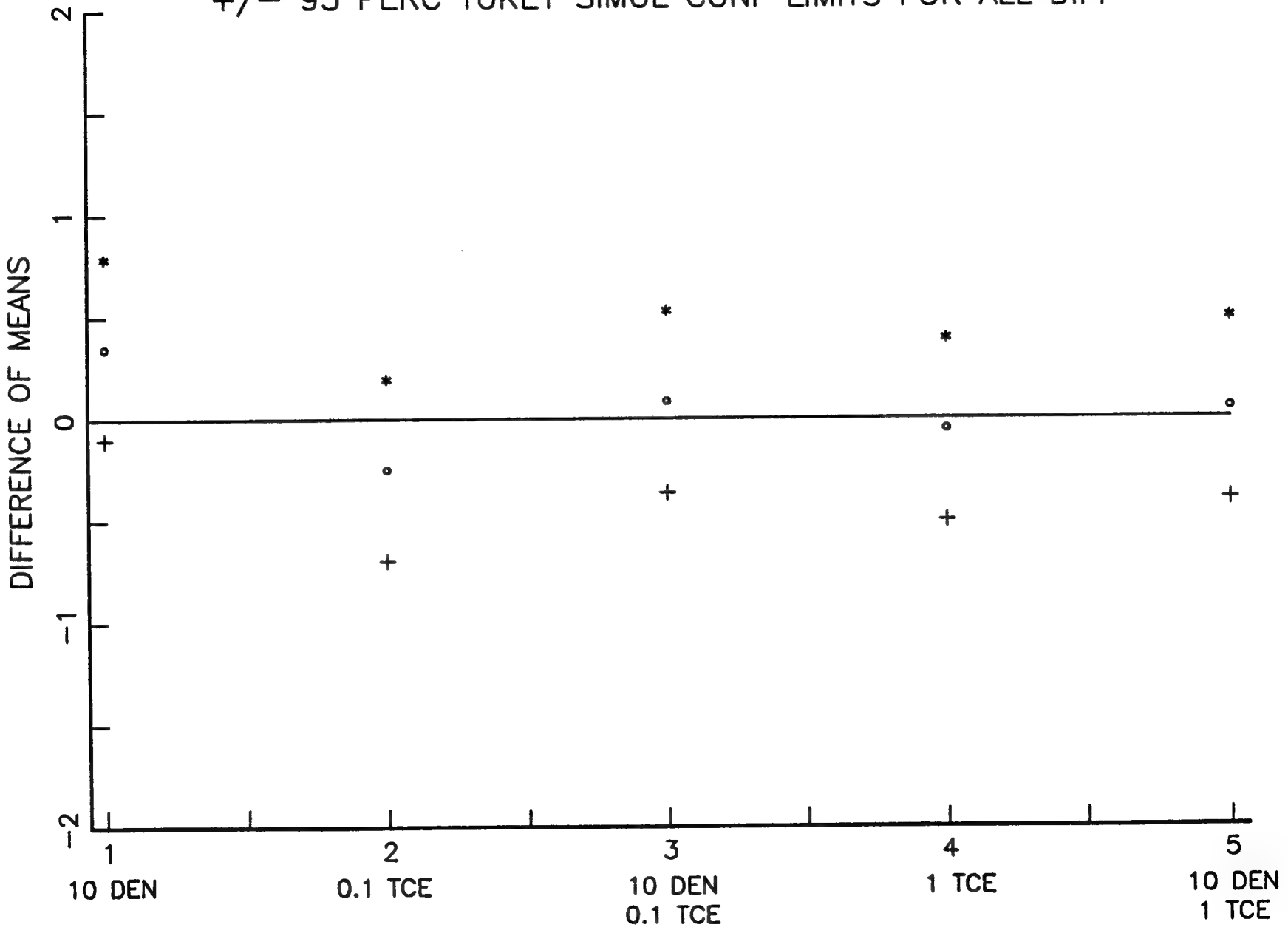
SACRIFICE D: SQUARE ROOT OF AREA INDICES
MEAN OF TREATMENT MEANS–MEAN OF CONTROL MEANS
+/– 95 PERC TUKEY SIMUL CONF LIMITS FOR ALL DIFF
DIFFERENCE OF MEANS
2
1
0
–1
–2
1
10 DEN
2
0.1 TCE
3
10 DEN
0.1 TCE
4
1 TCE
5
10 DEN
1 TCE

Figure 8
32

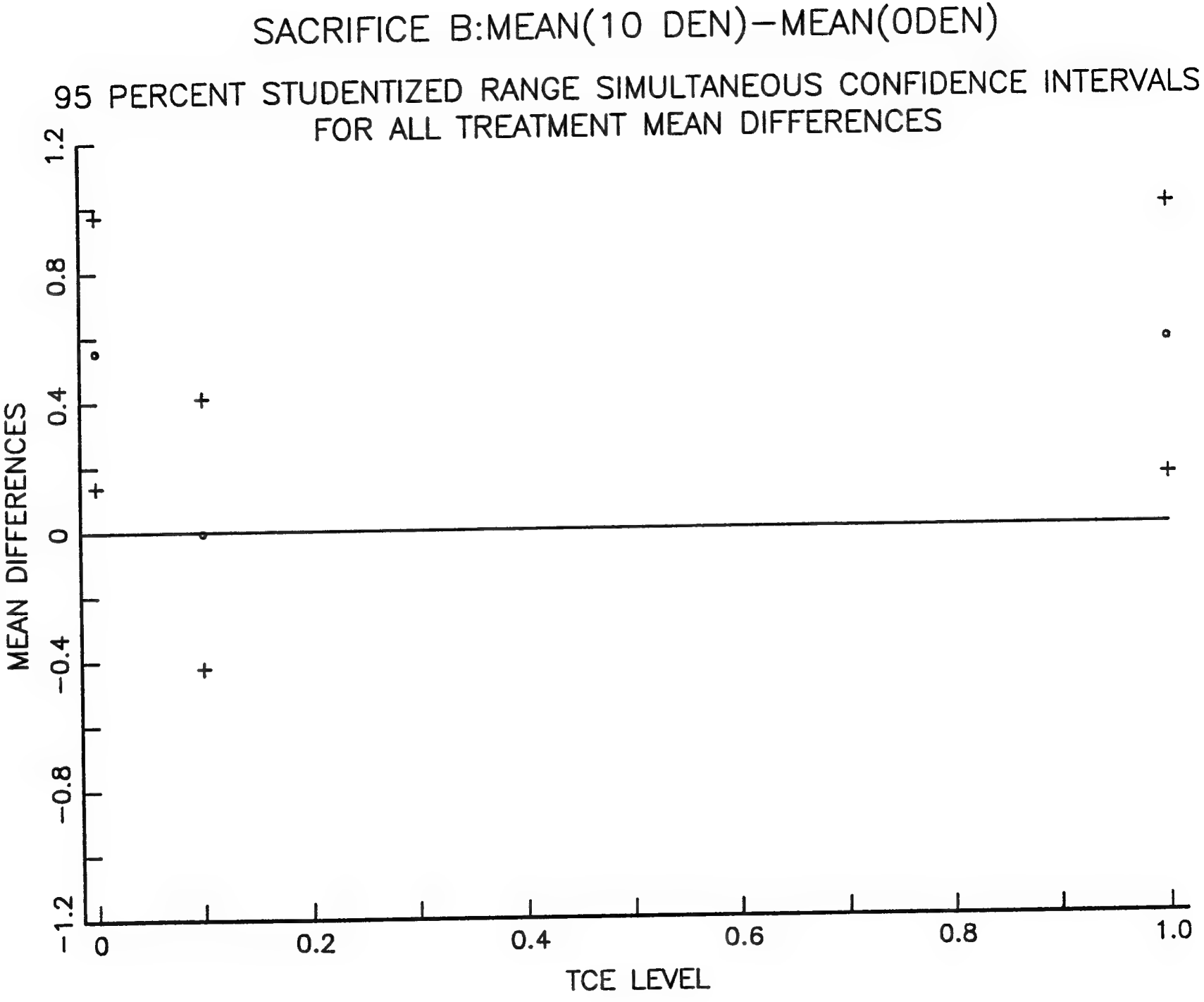
SACRIFICE B:MEAN(10 DEN)−MEAN(0DEN)
95 PERCENT STUDENTIZED RANGE SIMULTANEOUS CONFIDENCE INTERVALS
FOR ALL TREATMENT MEAN DIFFERENCES
MEAN DIFFERENCES
1.2
0.8
0.4
0
−0.4
−0.8
−1.2
0
0.2
0.4
0.6
0.8
1.0
TCE LEVEL

Figure 9

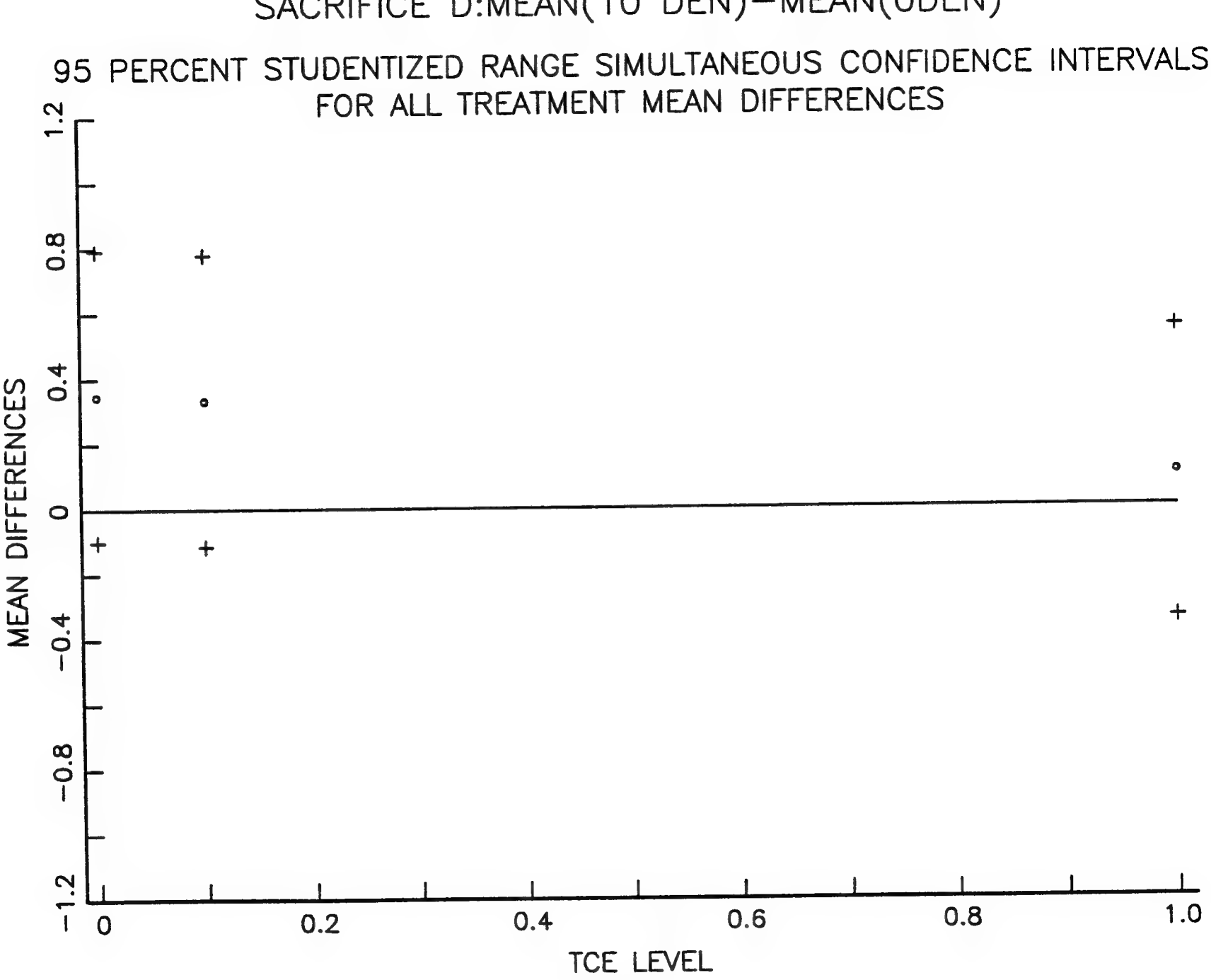
SACRIFICE D:MEAN(10 DEN)−MEAN(0DEN)
95 PERCENT STUDENTIZED RANGE SIMULTANEOUS CONFIDENCE INTERVALS
FOR ALL TREATMENT MEAN DIFFERENCES
MEAN DIFFERENCES
1.2
0.8
0.4
0
−0.4
−0.8
−1.2
0
0.2
0.4
0.6
0.8
1.0
TCE LEVEL

Figure 10

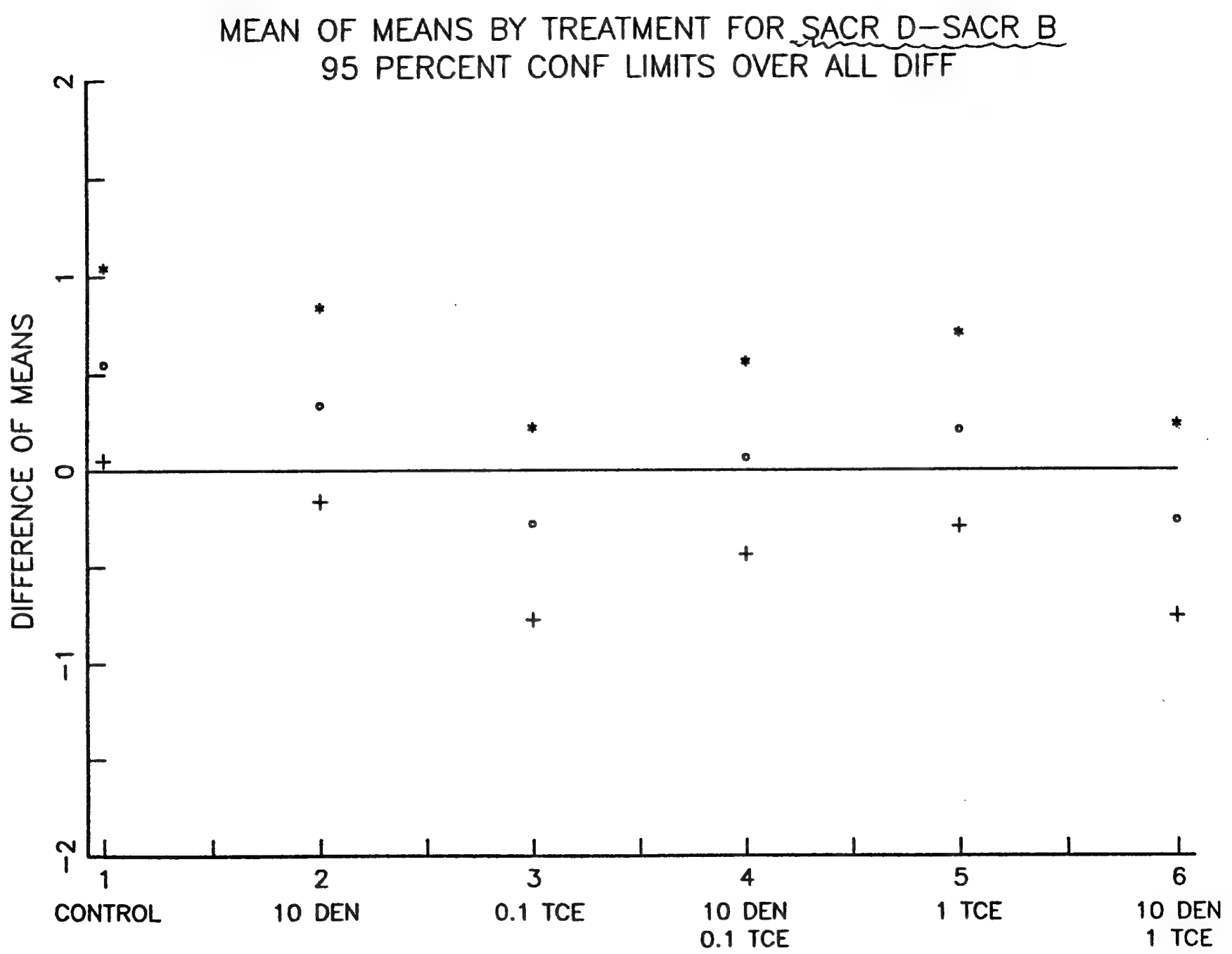
SQUARE ROOT OF AREA INDICES
MEAN OF MEANS BY TREATMENT FOR SACR D–SACR B
95 PERCENT CONF LIMITS OVER ALL DIFF
DIFFERENCE OF MEANS
2
1
0
−1
−2
1
CONTROL
2
10 DEN
3
0.1 TCE
4
10 DEN
0.1 TCE
5
1 TCE
6
10 DEN
1 TCE

Figure 11

SACRIFICE B:10 DEN; SQUARE ROOT OF AREA INDICES
STUDENTIZED MAXIMUM MODULUS SIMULTANEOUS CONFIDENCE INTERVALS
95 PERCENT
MEAN OF MEANS
0
0.5
1.0
1.5
2.0
2.5
LEVEL OF TCE
0
0.2
0.4
0.6
0.8
1.0

Figure 12

SACRIFICE D:10 DEN; SQUARE ROOT OF AREA INDICES
STUDENTIZED MAXIMUM MODULUS SIMULTANEOUS CONFIDENCE INTERVALS
95 PERCENT
MEAN OF MEANS
0
0.5
1.0
1.5
2.0
2.5
LEVEL OF TCE
0
0.2
0.4
0.6
0.8
1.0

Figure 13

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